

Basic Study

Thalidomide ameliorates portal hypertension *via* nitric oxide synthase independent reduced systolic blood pressure

Nicholas G Theodorakis, Yining N Wang, Vyacheslav A Korshunov, Mary A Maluccio, Nicholas J Skill

Nicholas G Theodorakis, Mary A Maluccio, Nicholas J Skill, Division of Transplant Surgery, Department of Surgery, School of Medicine, Indiana University, Indianapolis, IN 46202, United States

Yining N Wang, Vyacheslav A Korshunov, School of Medicine, University of Rochester, Rochester, NY 14611, United States

Author contributions: Theodorakis NG, Wang YN, Korshunov VA and Skill NJ performed the research; Skill NJ and Theodorakis NG designed the research; Skill NJ and Maluccio MA wrote the paper.

Supported by The Departments of Surgery at the University of Rochester and Indiana University.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Nicholas J Skill, PhD, Assistant Professor, Division of Transplant Surgery, Department of Surgery, School of Medicine, Indiana University, C519, 980 West Walnut Street (R3), Indianapolis, IN 46202, United States. nskill@iupui.edu

Telephone: +1-317-2744532

Fax: +1-317-2748046

Received: June 12, 2014

Peer-review started: June 12, 2014

First decision: July 9, 2014

Revised: July 30, 2014

Accepted: October 15, 2014

Article in press: October 15, 2014

Published online: April 14, 2015

Abstract

AIM: Portal hypertension is a common complication of liver cirrhosis and significantly increases mortality and morbidity. Previous reports have suggested that the compound thalidomide attenuates portal hypertension

(PHT). However, the mechanism for this action is not fully elucidated. One hypothesis is that thalidomide destabilizes tumor necrosis factor α (TNF α) mRNA and therefore diminishes TNF α induction of nitric oxide synthase (NOS) and the production of nitric oxide (NO). To examine this hypothesis, we utilized the murine partial portal vein ligation (PVL) PHT model in combination with endothelial or inducible NOS isoform gene knockout mice.

METHODS: Wild type, inducible nitric oxide synthase (iNOS)^{-/-} and endothelial nitric oxide synthase (eNOS)^{-/-} mice received either PVL or sham surgery and were given either thalidomide or vehicle. Serum nitrate (total nitrate, NOx) was measured daily for 7 d as a surrogate of NO synthesis. Serum TNF α level was quantified by enzyme-linked immunosorbent assay. TNF α mRNA was quantified in liver and aorta tissue by reverse transcription-polymerase chain reaction. PHT was determined by recording splenic pulp pressure (SPP) and abdominal aortic flow after 0-7 d. Response to thalidomide was determined by measurement of SPP and mean arterial pressure (MAP).

RESULTS: SPP, abdominal aortic flow (Qao) and plasma NOx were increased in wild type and iNOS^{-/-} PVL mice when compared to sham operated control mice. In contrast, SPP, Qao and plasma NOx were not increased in eNOS^{-/-} PVL mice when compared to sham controls. Serum TNF α level in both sham and PVL mice was below the detection limit of the commercial ELISA used. Therefore, the effect of thalidomide on serum TNF α levels was undetermined in wild type, eNOS^{-/-} or iNOS^{-/-} mice. Thalidomide acutely increased plasma NOx in wild type and eNOS^{-/-} mice but not iNOS^{-/-} mice. Moreover, thalidomide temporarily (0-90 min) decreased mean arterial pressure, SPP and Qao in wild type, eNOS^{-/-} and iNOS^{-/-} PVL mice, after which time levels returned to the respective baseline.

CONCLUSION: Thalidomide does not reduce portal

pressure in the murine PVL model by modulation of NO biosynthesis. Rather, thalidomide reduces PHT by decreasing MAP by an undetermined mechanism.

Key words: Portal hypertension; Thalidomide; Nitric oxide; Knockout mice; Endothelial nitric oxide synthase; Inducible nitric oxide synthase; Tumor necrosis factor alpha

© **The Author(s) 2015.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The research innovation detailed in this manuscript is the use of nitric oxide synthase (NOS) isoform specific gene deleted mice to better understand the underlying mechanisms for the development of portal hypertension (PHT). PHT is a significant complication of liver disease and increases morbidity and mortality. Our study examined the hypothesis that the compound thalidomide reduces PHT by decreasing the biosynthesis of nitric oxide (NO) *via* destabilizing tumor necrosis factor alpha mRNA levels. We demonstrate that thalidomide induces NO *via* increased inducible nitric oxide synthase isoform of NOS; however, thalidomide reduction in PHT was NOS isoform independent.

Theodorakis NG, Wang YN, Korshunov VA, Maluccio MA, Skill NJ. Thalidomide ameliorates portal hypertension *via* nitric oxide synthase independent reduced systolic blood pressure. *World J Gastroenterol* 2015; 21(14): 4126-4135 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i14/4126.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i14.4126>

INTRODUCTION

Portal hypertension (PHT) is a complication typically associated with underlying liver disease whereby portal pressure exceeds 10 mmHg^[1]. In general, PHT is predominantly a sequela of alcoholic, non-alcoholic steatohepatitis or viral cirrhosis^[2] and is augmented by the formation of a systemic hyper-dynamic circulation manifesting as a generalized reduced vascular resistance, *via* vasodilation, promoting systemic and mesenteric hyperemia^[3,4]. Increased hepatic resistance in combination with hyperemia promotes redirection of portal venous flow from the liver towards, *inter alia*, mesenteric and azygous venous beds to form esophageal and ectopic varices^[5]. Generally, esophagogastric varices have the greatest capacity for hemorrhage amongst varices and in combination with liver failure significantly increase mortality and morbidity^[6].

Previous studies have detailed the importance of nitric oxide synthase (NOS) enzymes in the development of PHT. In particular, the NOS isoform endothelial NOS (eNOS) is thought to be important to PHT by regulating biosynthesis of the potent vasodilator nitric oxide

(NO)^[7-9]. Decreased hepatic eNOS results in hepatic sinusoid constriction and increased hepatic resistance to portal venous flow^[10]. In contrast, increased extra-hepatic vascular eNOS promotes vasodilation and the development of hyper-dynamic circulation by increasing blood flow to the liver^[11,12]. Moreover, functional eNOS is also found in circulating micro-particles (< 1 µm) shed from endothelial and blood cells. Studies show that micro-particles are increased in patients with endothelial dysfunction but eNOS expression is decreased^[13]. Consequently, the precise mechanism/role for eNOS in PHT is not clear. We do know that eNOS activation is multifaceted and includes protein kinase B AKT, GTPase-activating protein, sarcoma proto-oncogene and G protein-coupled receptor-2 kinase activity culminating with eNOS phosphorylation and translocation to the cell membrane^[8,14-16].

In addition, the cytokine tumor necrosis factor-alpha (TNF-α) is thought to be important in the pathogenesis of PHT by increasing eNOS activity and NO production^[17,18]. Therefore, inhibition of TNFα has the potential to ameliorate portal pressure and reduce mortality and morbidity associated with PHT. Previous studies have shown that the compound thalidomide destabilizes TNFα mRNA^[19] and prevents the development of PHT in portal vein ligated (PVL) animals^[20,21]. However, the precise mechanism by which thalidomide abrogates PHT is not fully understood. In human studies, thalidomide reduces hepatic venous pressure gradient without reducing systemic hemodynamic parameters^[22]. This suggests that: (1) TNFα is not associated with PHT associated hyper-dynamic circulation; and (2) thalidomide reduces PHT independent of TNFα and NO.

To examine the mechanistic connection between thalidomide, TNFα and NO on PHT, we utilized the murine, non-inflammatory, pre-hepatic PVL model of PHT and commercially available iNOS and eNOS gene knockout mice and examined changes to TNFα and PHT in response to thalidomide. We have previously shown that in the PVL model of PHT, eNOS is the dominant NOS isoform and in its absence PHT does not develop^[9]. We anticipated that in PVL mice, TNFα would be increased and that thalidomide would reduce this increase and associated PHT but there would be no response to thalidomide in eNOS^{-/-} mice. In contrast to this expectation, we found that TNFα levels were below detectable limits of the enzyme-linked immunosorbent assay (ELISA) used and that thalidomide caused: (1) an iNOS dependent increase in circulating NOx levels; and (2) thalidomide reduction of portal pressure was NOS isoform independent and was commensurate with a NOS independent drop in systolic blood pressure. Because TNFα levels in mice were below detectable levels, we were unable to determine response to thalidomide administration. Liver and thoracic aorta tissue TNFα mRNA expression levels were detectable and levels were not significantly

changed in response to PVL surgery but were increased following thalidomide administration. This information demonstrates that thalidomide improves portal hemodynamics independent of NO and is linked to a reduction in mean arterial blood pressure.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma, MO. The partial portal vein ligation pre-hepatic PHT model was used. All studies were approved by the University of Rochester committee for animal research and adhered to AAALC and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolette cages on a 12-h light/dark cycle and were allowed access to food and water *ad libitum*. Mice [C57B/6J, C57BL/6J-NOS2^{tm1Unc} (iNOS^{-/-})^[23] and C57BL/6J-NOS3^{tm1Unc} (eNOS^{-/-})^[24] (Jackson Labs, MA)] were anesthetized using halothane inhalation. A midline laparotomy was performed and the portal vein was exposed. A blunt-ended 27-gauge needle was placed alongside the portal vein and a 4-0 silk suture was tied around the vein and needle, after which the needle was withdrawn, producing a standardized stenosis. In sham animals, the procedure consisted of dissection and visual inspection of the portal vein without ligation. The abdomen was closed and the animals were allowed to recover under a heat lamp. For thalidomide studies, (25-100 mg/kg per day) thalidomide (α -N-phthalylglutamic-acid-imine) or dimethyl sulfoxide (DMSO) vehicle was given 16 h prior and 4 h following PVL or sham and every 24 h thereafter.

Physiological measurements

Physiological measurements were performed as previously described^[9]. At the indicated times post sham-operation or PVL, animals were anesthetized (halothane) and subjected to laparotomy to allow physiological measurements to be taken. Splenic pulp pressure (SPP) was measured as an index of portal venous pressure. To measure SPP, a cannula made from a 25-gauge needle connected to a saline-filled manometer was inserted into the spleen pulp. Abdominal aortic flow was measured by placing an ultrasonic Doppler flow probe (Transonic #11RB) around the abdominal aorta between the diaphragm and celiac artery. Flow rates were obtained with a Transonic T206 Blood Flow Meter (Transonic Instruments, Ithaca, NY). Aortic blood flows were standardized per gram of body weight.

Systolic blood pressure and heart rate were determined by non-invasive tail cuff plethysmography using the Visitech BP-2000 Blood Pressure Analysis system as per manufacturer's instructions (Visitech systems, Apex, NC). Baseline values were obtained for 4 d to train the mice ($n = 8$) in the measurement of blood pressure and heart rate. On the fifth day, mice were

given 50 mg/kg thalidomide and the blood pressure and heart rate was calculated for 10 min every 20 min.

Plasma TNF α and NOx levels

Blood was collected by cardiac puncture, injected into heparinized tubes and centrifuged. Plasma TNF α was measured by sandwich ELISA in accordance with manufacturer's instructions (#MTA00B, RD systems, Minneapolis, MN). TNF α was measured 0, 1, 3, 6, 12, 16, 20 and 24 h and 2, 3, 4, 5, 6 and 7 d following PVL operation. Plasma NOx was determined using the Griess reaction^[25] using a commercially available kit (#NB98, Oxford Biomedical, Rochester Hills, MI).

In vitro cell culture: RAW264 mouse macrophage cells (#TIB-71 ATCC, Manassas, VI) were cultured in the presence or absence of LPA and/or 25-100 μ g/mL thalidomide for 0-24 h.

Gene expression: TNF α , eNOS and iNOS mRNA from liver, aorta or RAW264 cells were determined by reverse transcription-polymerase chain reaction (RT-PCR) using gene-specific primers (200 ng) and 1 μ g cDNA: eNOS: 5'GTGTGAAGGCAACCATCTG 3'ACTCATCCATG CACAGGACC, iNOS: 5'GGCTTCACGGGTCAGAGCCA 3'TGCCCATTCG TGGGACAGTC TNF α 5'CTGTAGCC-CACGTCGTAGC 3'TTGAGATCCATGCCGTTG 3 (cycle = 1 min each of 94 oc, 60 oc and 74 oc \times 35). Primers were purchased from Life Technologies, Grand Island, NY.

Statistical analysis

The data shown are mean \pm SE, with 3-7 animals per experimental group. Statistical significance was estimated using ANOVA statistical analysis (SPSS, IBM).

RESULTS

TNF- α and NOx levels following PVL or thalidomide administration: plasma TNF α and NOx levels were determined following sham or PVL ligation and after thalidomide injection. TNF α levels were below the detectable levels of the assay. Consequently, no increase was detected following PVL and no decrease following thalidomide injection. Injection of 1 mg/kg LPS increased serum TNF α from undetectable levels to 192 pg/mL. Plasma NOx was increased significantly following PVL in wild type mice, peaking at 2 d post PVL surgery (Figure 1A). This PVL induced NOx at 2 d post-surgery was eNOS specific. NOx was not increased in eNOS^{-/-} mice following PVL but was increased in iNOS^{-/-} mice (Figure 1B). Thalidomide induction of NOx was iNOS specific. Serum NOx was not increased in iNOS^{-/-} mice following thalidomide injection but was increased in eNOS^{-/-} mice (Figure 1C). Thalidomide increased iNOS mRNA levels in aorta and liver tissue samples (75% and 162% respectively at 100 mg/kg). No change in eNOS expression was

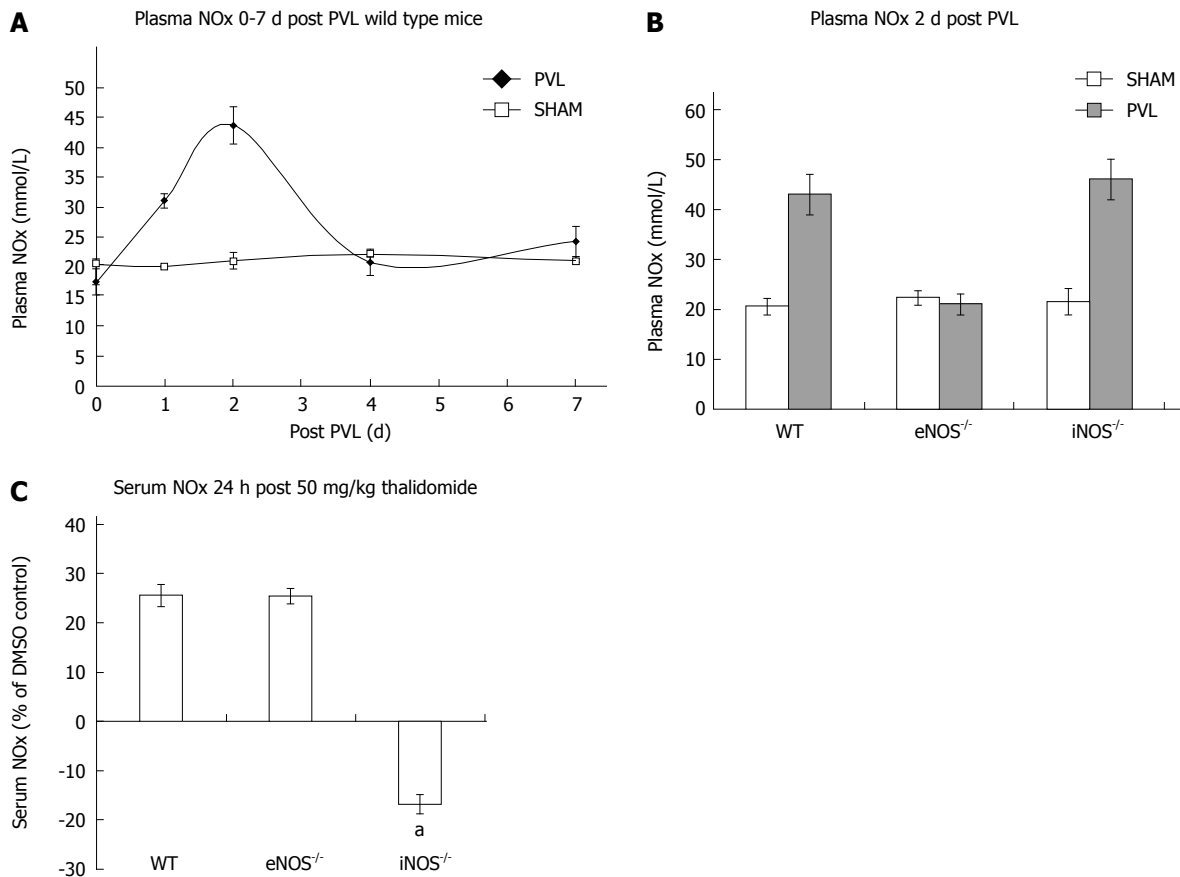


Figure 1 Portal vein ligation and thalidomide increases nitric oxide via endothelial and inducible nitric oxide synthase respectively. Plasma total nitrate (NOx) was measured daily for 7 d in wild type (WT), endothelial nitric oxide synthase (eNOS)^{-/-} and inducible nitric oxide synthase (iNOS)^{-/-} mice following portal vein ligation (PVL) or sham surgery. A: Plasma NOx increased following PVL in wild type mice but not following sham surgery. Levels increased to a maximum 2 d after PVL, after which NOx returned to pre-surgical baseline; B: Plasma NOx increased 2 d following PVL in iNOS^{-/-} but not eNOS^{-/-} mice. In eNOS^{-/-} mice NO was not increased following PVL; C: Unadulterated 8 wk WT, eNOS^{-/-} and iNOS^{-/-} mice were given 50 mg/kg thalidomide or vehicle. Blood was collected by cardiac puncture and plasma was assayed for NOx. Plasma NOx was significantly increased by the administration of thalidomide in WT and eNOS^{-/-} mice but was reduced in iNOS^{-/-} mice. ^a*P* < 0.05 vs other groups.

Table 1 Splenic pulp pressure and abdominal aortic flow 7 d post portal vein ligation or sham

	Splenic pulp pressure (cmH ₂ O)	Abdominal aortic flow (mL/min per gram)
Wild type sham	6.9 ± 0.4	0.17 ± 0.02
Wild type 7 d PVL	25.4 ± 3.1 ^a	0.27 ± 0.04 ^a
eNOS ^{-/-} sham	6.9 ± 0.3	0.15 ± 0.01
eNOS ^{-/-} 7 d PVL	7 ± 0.5	0.15 ± 0.01
iNOS ^{-/-} 7 d sham	6.7 ± 1	0.16 ± 0.01
iNOS ^{-/-} 7 d PVL	21.1 ± 0.4 ^c	0.23 ± 0.06 ^c

^a*P* < 0.05 vs wild type sham *t*-test; ^c*P* < 0.05 vs iNOS^{-/-} 7d sham *t*-test; mean ± SE (*n* = 4-7). iNOS: Inducible nitric oxide synthase.

observed.

Hepatic and arterial TNF α , eNOS and iNOS expression following thalidomide administration: thalidomide is reported to destabilize TNF α mRNA^[19]. In immortalized macrophage cells (RAW263.3), we found that thalidomide reduced TNF α mRNA in unstimulated and LPS stimulated cells (81.7% and 78.6% respectively). In contrast, thalidomide did not reduce LPS stimulated iNOS mRNA induction.

The effect of thalidomide *in vivo* was determined by quantification of hepatic and arterial tissue TNF α , eNOS and iNOS expression \pm thalidomide by RT-PCR. TNF α mRNA was increased by 62% in thoracic aorta tissues and 34% in the liver tissues. In a similar manner, hepatic iNOS was increased 70% and 49% respectively. No change in eNOS mRNA was observed.

Effects of thalidomide on mean blood pressure, heart rate and portal hemodynamics following PVL: in vehicle treated mice, splenic pulp pressure and abdominal aortic flow were increased significantly 7 d following PVL (Table 1). Treatment of mice with 50 mg/kg thalidomide resulted in a temporary (90 min) reduction in elevated splenic pressure and mean systolic blood pressure (58% and 70% reduction respectively) (Figure 2A and B). Thalidomide induced reduction was maximal 30-60 min following injection and after which time splenic pulp pressure and mean arterial pressure recovered to an elevated level by 90 min. No significant change in heart rate was observed following thalidomide administration in PVL or sham mouse groups.

Role of NOS isoforms on thalidomide: to determine

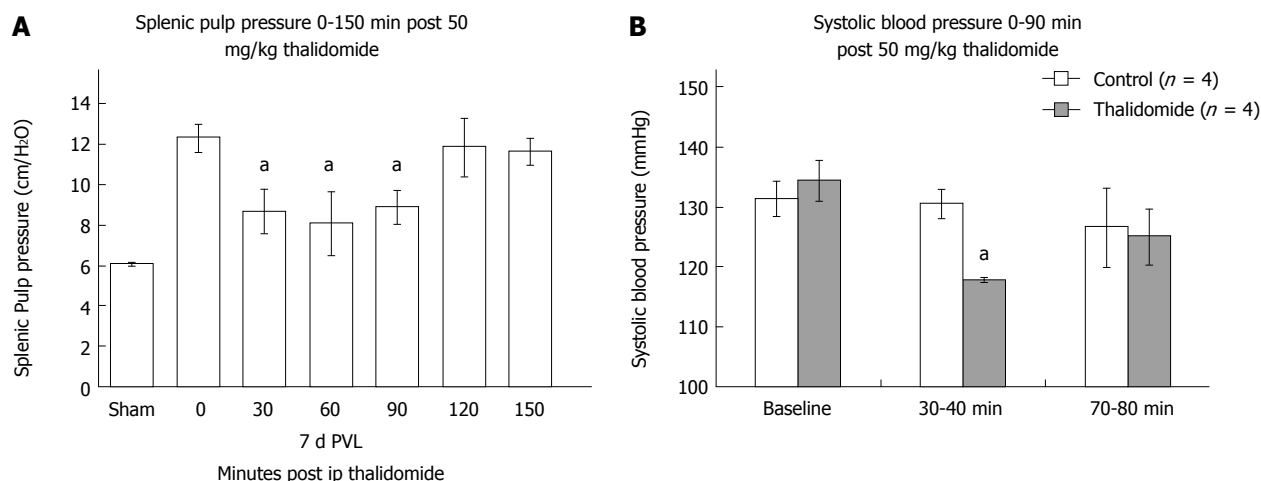


Figure 2 Thalidomide temporarily ameliorates splenic pulp pressure and mean systolic blood pressure in 7 d portal vein ligation mice. A: 7 d wild type sham and portal vein ligation (PVL) mice were treated with 50 mg/kg thalidomide *ip* and splenic pulp pressure was measured 0-150 min following administration. Splenic pulp pressure was rapidly and temporarily decreased by thalidomide; after 2 h pressure returned back to pre-thalidomide levels; B: Systolic blood pressure was also measured in 7 d PVL wild type mice following the administration of 50 mg/kg thalidomide or vehicle control. In a similar manner to splenic pulp pressure the systolic blood pressure was temporarily decreased by thalidomide. ^a*P* < 0.05 vs control group.

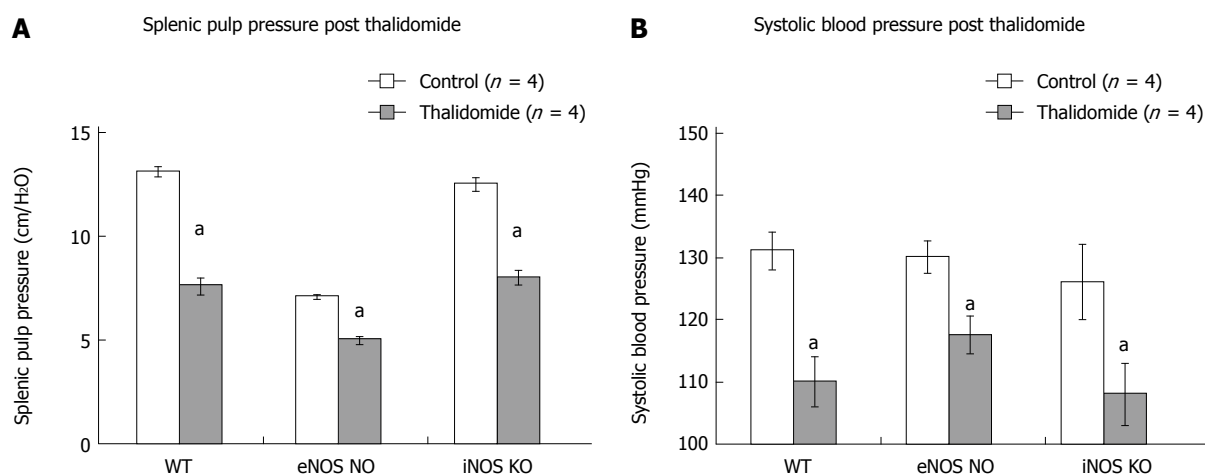


Figure 3 Thalidomide reduction of splenic pulp pressure and systolic blood pressure is endothelial and inducible nitric oxide synthase independent. 7 d portal vein ligation wild type, endothelial nitric oxide synthase (eNOS)^{-/-} and inducible nitric oxide synthase (iNOS)^{-/-} mice were treated with 50 mg/kg thalidomide or dimethyl sulfoxide vehicle control *ip*. Splenic pulp pressure (A) and systolic blood pressure (B) were measured 60 min after administration. Thalidomide reduced splenic pulp pressure and systolic blood pressure in wild type, eNOS^{-/-} and iNOS^{-/-} mice (*n* = 4 mice per group, ^a*P* < 0.05 vs control group).

the role of NOS isoforms on the transient hemodynamic response to thalidomide, wild type, eNOS^{-/-} and iNOS^{-/-} 7 d PVL mice were treated with 50 mg/kg thalidomide and hemodynamic measurements were performed 30 min thereafter. Portal pressure and systolic blood pressure were significantly reduced in all mice groups (Figure 3). Reduction in hemodynamics in response to thalidomide was NOS isoform independent.

TNF α aortic and hepatic expression post PVL: to evaluate the role of TNF α in the PVL model, levels were determined by RT-PCR in aorta and liver tissue samples 0-4 d following PVL. TNF α was generally unchanged in response to PVL in both liver and aorta samples. However, there was a transient reduction in TNF α expression 3 h post PVL within aortic tissues but this reduction was not significantly significant (*P* = 0.1) (Figure 4). Whole tissues were used and so

differentiation between muscle, nervous, connective and epithelial tissue was not investigated.

DISCUSSION

In 1998, the US Food and Drug Administration approved the use of thalidomide for the treatment of leprosy and multiple myeloma. Additional research has investigated the benefit of thalidomide in animal models of Alzheimer's^[26], pancreatitis^[27], colitis^[28] and PHT^[20].

The purpose of this manuscript is not to advocate the use of thalidomide for the treatment of PHT. That claim is beyond the scope of our experimental studies and we leave that analysis for others. Using the PVL murine model of pre-hepatic PHT in eNOS^{-/-} and iNOS^{-/-} deficient animals, this study investigates thalidomide abrogation of PHT *via* mediating activation of NO

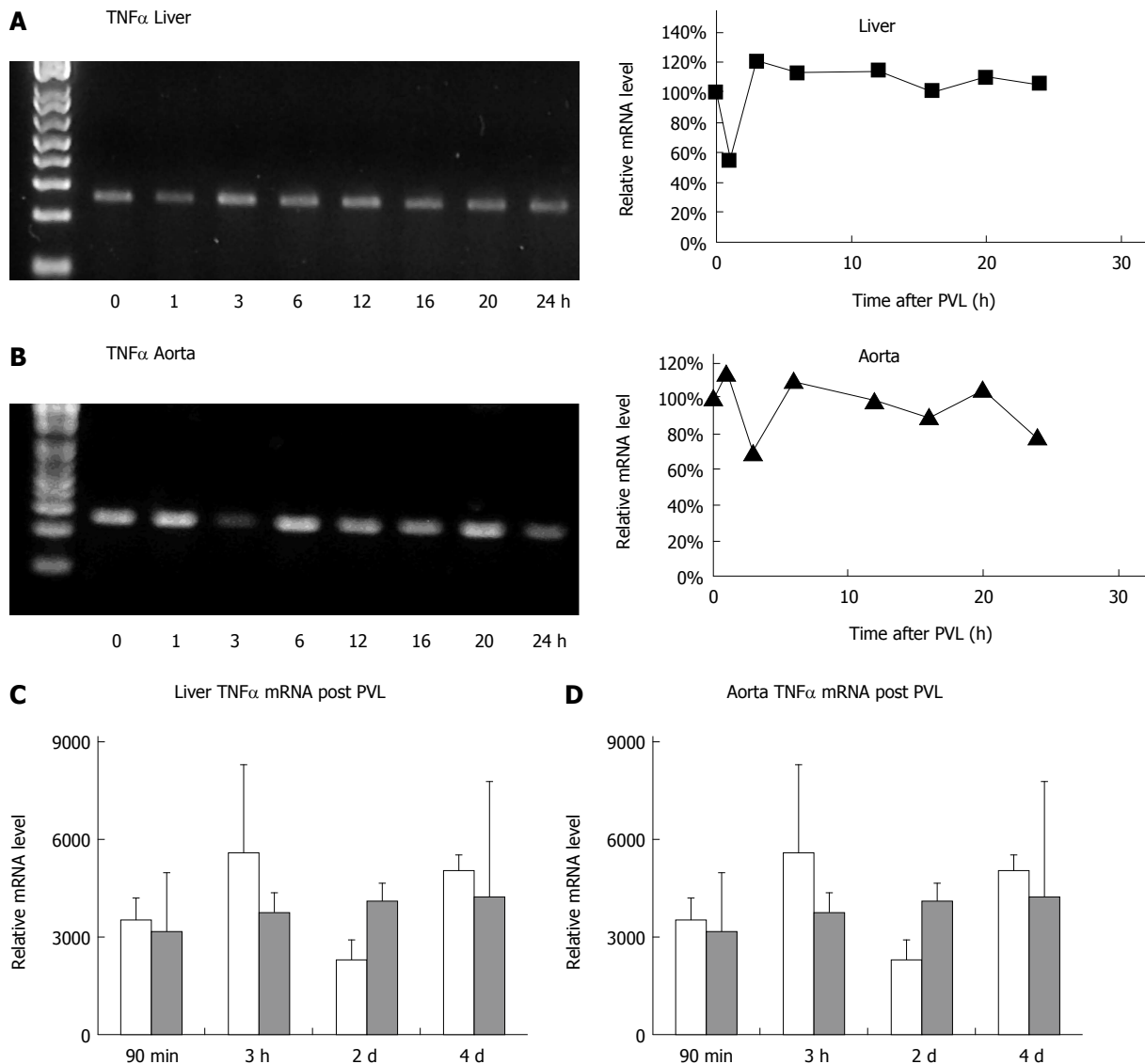


Figure 4 Portal vein ligation does not increase hepatic or aortic tumor necrosis factor alpha mRNA expression. Pre-hepatic portal hypertension was induced in wild type mice by partial ligation of the portal vein. 0-24 h post ligation livers (A) and thoracic aortas (B) were harvested and quantified for tumor necrosis factor (TNF) mRNA by reverse transcription-polymerase chain reaction. TNF α was not changed within livers and aorta following portal vein ligation (PVL). Figures are representative of three experiments. Hepatic (C) and aortic (D) TNF α expression was unchanged 0-4 d post PVL. Line graphs represent mean \pm SE. $n = 5$ mice.

production. Our data shows that thalidomide increases NO production, *via* induction of iNOS, but this increase is not important to thalidomide transient reduction of PHT. Moreover, given that TNF α expression in liver and aorta tissues was unchanged in response to PVL, the murine PVL model is not suitable to study the role of TNF α in PHT. However, the demonstration that thalidomide temporarily reduces mean systemic blood pressure by an NO independent mechanism illustrates that thalidomide may be beneficial to prevent variceal formation and hemorrhage, a common problem associated with PHT, especially in patients that are non-responsive to current β -blocker therapy to reduce systemic blood pressure^[29].

The scientific consensus is that the vasodilator NO plays a major role in the development and sustained vasculopathy of PHT^[4]. However, the mechanisms

controlling NO biosynthesis in the context of PHT are not clearly defined. Previous investigations using animal models of PHT and human studies suggest a role for TNF- α in vasodilatation by promoting increased NO production^[30] by arguing that TNF- α induction of NOS isoforms (eNOS, and/or iNOS) promotes the development of a hyper-dynamic circulation and vascular hypo-responsiveness to vasoconstrictors associated with this pathological dysfunction^[31]. Therefore, inhibition of TNF α is a potential mechanism in which to reduce PHT. Previous studies have shown that the compound thalidomide reduces TNF α ^[8,32]. The purpose of this manuscript was to investigate the hypothesis that TNF α modulates PHT *via* nitric oxide synthase enzyme^[33]. We found that thalidomide, rather than reduce NO levels, increased circulating plasma NOx levels *via* iNOS. Moreover, although

thalidomide does reduce TNF α mRNA levels in mouse macrophage cells (RAW286.3), thalidomide increased TNF α and iNOS mRNA expression in murine liver and thoracic aorta tissues, suggesting that *in vivo* thalidomide increases TNF α and iNOS levels. It is not unexpected that thalidomide would modulate TNF α and iNOS similarly as co-regulation of TNF α and iNOS in response to stimuli is well described^[34,35], as is the interaction between TNF α expression and iNOS expression^[36,37]. Moreover, TNF α neutralizing antibodies are known to reduce iNOS expression^[38-40].

Despite detecting an increase in TNF α expression, we were unable to detect circulating TNF α in sham or PVL mice irrespective of thalidomide administration using a highly specific commercially available ELISA, arguing against a role for TNF α in PVL models of PHT. However, studies by others have detected TNF α in rodent models of PVL using biological cell based assays^[20]. The fact that TNF α neutralizing antibodies reduce NO and portal pressure in PVL rats suggests that TNF α is important in the PVL model *via* modulation of NOS^[30]. Although TNF α levels were undetectable, the effects of thalidomide on NOS isoforms can be determined by measuring circulating NO levels, hemodynamic changes and TNF α mRNA levels.

In rat PHT models, thalidomide administration demonstrated a significant correlation between TNF α plasma levels and mean arterial pressure among PVL animals^[20]. Thalidomide has been noted to have many possible vascular effects, including anti-angiogenesis^[41], disruption of mRNA transcription causing attenuation of the nuclear factor kappa B mediated gene expression^[42], increase of the production of free radicals to elicit oxidative stress^[43] and directly through systemic circulatory and/or direct cardiac effects^[44]. Consequently, a therapeutic role for thalidomide in patients with cirrhosis has been proposed^[22]. We found that thalidomide reduced PHT *via* a significant reduction in mean arterial pressure (MAP) that was NOS isoform independent and irrelevant to circulating NO levels. MAP is approximately determined from measurements of the systolic pressure and the diastolic pressure over a cardiac cycle and is determined by the cardiac output (CO), systemic vascular resistance (SVR) and central venous pressure [MAP = (CO - SVR) + CVR]. Cardiac output is related to both heart rate and stroke volume (SV). Both thalidomide and TNF α have been linked to vascular regulation.

Thalidomide has been shown to improve CO by increasing the left ventricular ejection fraction^[44,45] and causes an imbalance between vasodilators (NO) and vasoconstrictors (endothelin-1) that impacts SVR^[46]. Our data suggests that modulation of NO is linked to iNOS. Studies by others have reported that thalidomide does not change endothelin-1 levels in human endothelial cells^[47]. While the vascular response to thalidomide was not the original focus of this study, we did observe that heart rate was not affected by thalidomide. This suggests changes in SVR, CVR or

SV. TNF α has been shown to directly increase cardiac index and mean arterial pressure, systemic vascular resistance index, temperature, and heart rate^[48,49]. However, because there appears to be no discernable increase in TNF α within the PVL murine model of PHT, other models are required to fully understand the connections amongst TNF α , thalidomide and PHT.

In conclusion, although previous reports demonstrate the importance of TNF α in the development of PHT, TNF α does not appear to be important in the murine PVL model. This correlates with our previous study demonstrating that gene deletion of TNF α receptors does not ameliorate PHT in mice following PVL^[50]. Consequently, the PVL model of PHT is not conducive to the study of TNF α in PHT. Despite this lack of TNF α involvement, thalidomide treatment demonstrated a temporary reduction in PHT *via* a NOS isoform independent mechanism, indicating a non-TNF α and non-NO mechanism for thalidomide in PHT. While this observation is interesting because thalidomide may be beneficial for the treatment of PHT associated variceal formation and bleeding, the use of thalidomide is cautioned since thalidomide is associated with thromboembolic events that would exacerbate PHT and enhance liver damage^[51]. However, use of thalidomide analogs may demonstrate therapeutic benefit without vascular complications^[52]. Further investigation is required to better understand the mechanism by which thalidomide reduces portal pressure with the anticipation that advances can be translated to clinical practice and improve outcomes for patients with liver disease and a high risk of developing PHT. We do not suggest by what mechanism thalidomide reduces mean arterial blood pressure because thalidomide is linked to many biological mechanisms, including vascular endothelial growth factor and thromboxane, both of which have been linked to PHT^[53-55]. Moreover, thalidomide is known to modulate PHT *via* cannabinoid receptor-2 (CB2) expression and studies show that targeting CB2 receptor agonists ameliorate PHT in bile duct ligated rats and that thalidomide increases CB2 receptor expression and reduces cannabinoid receptor-1 expression^[56,57].

ACKNOWLEDGMENTS

The authors would like to thank Dr. James Sitzmann and Dr. Brad Berk, University of Rochester School of Medicine, for fostering the completion of this work.

COMMENTS

Background

Portal hypertension is an elevation in the portal venous pressure and increases mortality and morbidity in patients. The compound thalidomide has been shown to reduce portal hypertension. However, the mechanism of this response is unclear and the use of thalidomide is controversial. Previous studies have demonstrated that thalidomide reduces biosynthesis of the potent vasodilator nitric oxide (NO) by destabilizing tumor necrosis factor alpha mRNA. The

purpose of this study was to test this hypothesis using mice that have targeted gene deletions affecting NO biosynthesis.

Research frontiers

There is a need to find alternative treatment paradigms for treating portal hypertension, including diagnosis, measurement and treatment. Thalidomide has been shown to reduce portal pressure and thalidomide derivatives are being explored. The hot spot is to better understand how these compounds work in order to facilitate translation in to the clinic.

Innovations and breakthroughs

The innovation of this research is the utilization of gene deleted mice and the microsurgery. In combination, this provides a cleaner understanding of the role of individual genes within disease pathology. The portal vein ligation model avoids the inflammatory and cytokine milieu associated with the carbon tetrachloride or bile duct ligation models of portal hypertension.

Applications

This study confirms that thalidomide does reduce portal hypertension but that this response is transient and last about 1 h. Moreover, the reduction is not linked to NO biosynthesis but *via* a nitric oxide synthase (NOS) independent reduction in mean systolic pressure. These results support the use of thalidomide or its derivatives and will direct further studies to investigate alternative targets other than NO.

Terminology

Portal hypertension is driven by two main pathologies and both are related to NO. Reduced hepatic NO biosynthesis causes sinusoidal constriction that increases resistance to portal flow and increases portal pressure. In contrast, NO is increased within the systemic vasculature, resulting in increased cardiac output and increased portal venous flow that increase portal pressure.

Peer-review

The authors examined the hypothesis that thalidomide diminishes tumor necrosis factor alpha induction of NOS and the production of NO. They concluded that the transitory reduction in portal pressure was associated with an inducible NOS dependent increase in NO and a NOS isoform independent reduction in blood pressure.

REFERENCES

- Nusrat S, Khan MS, Fazili J, Madhoun MF. Cirrhosis and its complications: evidence based treatment. *World J Gastroenterol* 2014; **20**: 5442-5460 [PMID: 24833875 DOI: 10.3748/wjg.v20.i18.5442]
- Sanyal AJ, Bosch J, Blei A, Arroyo V. Portal hypertension and its complications. *Gastroenterology* 2008; **134**: 1715-1728 [PMID: 18471549 DOI: 10.1053/j.gastro.2008.03.007]
- Vorobioff J, Bredfeldt JE, Groszmann RJ. Hyperdynamic circulation in portal-hypertensive rat model: a primary factor for maintenance of chronic portal hypertension. *Am J Physiol* 1983; **244**: G52-G57 [PMID: 6849394]
- Hu LS, George J, Wang JH. Current concepts on the role of nitric oxide in portal hypertension. *World J Gastroenterol* 2013; **19**: 1707-1717 [PMID: 23555159 DOI: 10.3748/wjg.v19.i11.1707]
- Al-Busafi SA, McNabb-Baltar J, Farag A, Hilzenrat N. Clinical manifestations of portal hypertension. *Int J Hepatol* 2012; **2012**: 203794 [PMID: 23024865 DOI: 10.1155/2012/203794]
- Sato M, Tateishi R, Yasunaga H, Horiguchi H, Yoshida H, Fushimi K, Koike K. Variceal hemorrhage: Analysis of 9987 cases from a Japanese nationwide database. *Hepatol Res* 2015; **45**: 288-293 [PMID: 24827451 DOI: 10.1111/hepr.12357]
- Heinemann A, Wachter CH, Holzer P, Fickert P, Stauber RE. Nitric oxide-dependent and -independent vascular hyporeactivity in mesenteric arteries of portal hypertensive rats. *Br J Pharmacol* 1997; **121**: 1031-1037 [PMID: 9222564 DOI: 10.1038/sj.bjp.0701220]
- Liu S, Rockey DC. Cicletanine stimulates eNOS phosphorylation and NO production via Akt and MAP kinase/Erk signaling in sinusoidal endothelial cells. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G163-G171 [PMID: 23639812 DOI: 10.1152/ajpgi.00003.2013]
- Theodorakis NG, Wang YN, Skill NJ, Metz MA, Cahill PA, Redmond EM, Sitzmann JV. The role of nitric oxide synthase isoforms in extrahepatic portal hypertension: studies in gene-knockout mice. *Gastroenterology* 2003; **124**: 1500-1508 [PMID: 12730888]
- Rockey DC, Chung JJ. Reduced nitric oxide production by endothelial cells in cirrhotic rat liver: endothelial dysfunction in portal hypertension. *Gastroenterology* 1998; **114**: 344-351 [PMID: 9453496]
- Cahill PA, Redmond EM, Hodges R, Zhang S, Sitzmann JV. Increased endothelial nitric oxide synthase activity in the hyperemic vessels of portal hypertensive rats. *J Hepatol* 1996; **25**: 370-378 [PMID: 8895017]
- Wiest R, Shah V, Sessa WC, Groszmann RJ. NO overproduction by eNOS precedes hyperdynamic splanchnic circulation in portal hypertensive rats. *Am J Physiol* 1999; **276**: G1043-G1051 [PMID: 10198349]
- Horn P, Cortese-Krott MM, Amabile N, Hundsdörfer C, Kröncke KD, Kelm M, Heiss C. Circulating microparticles carry a functional endothelial nitric oxide synthase that is decreased in patients with endothelial dysfunction. *J Am Heart Assoc* 2013; **2**: e003764 [PMID: 23525410 DOI: 10.1161/JAHA.112.003764]
- Liu S, Premont RT, Kontos CD, Zhu S, Rockey DC. A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nat Med* 2005; **11**: 952-958 [PMID: 16142243 DOI: 10.1038/nm1289]
- Liu S, Premont RT, Rockey DC. G-protein-coupled receptor kinase interactor-1 (GIT1) is a new endothelial nitric-oxide synthase (eNOS) interactor with functional effects on vascular homeostasis. *J Biol Chem* 2012; **287**: 12309-12320 [PMID: 22294688 DOI: 10.1074/jbc.M111.320465]
- Liu S, Premont RT, Rockey DC. Endothelial nitric-oxide synthase (eNOS) is activated through G-protein-coupled receptor kinase-interacting protein 1 (GIT1) tyrosine phosphorylation and Src protein. *J Biol Chem* 2014; **289**: 18163-18174 [PMID: 24764294 DOI: 10.1074/jbc.M113.521203]
- Kaviani A, Ohta M, Itani R, Sander F, Tarnawski AS, Sarfeh IJ. Tumor necrosis factor-alpha regulates inducible nitric oxide synthase gene expression in the portal hypertensive gastric mucosa of the rat. *J Gastrointest Surg* 1997; **1**: 371-376 [PMID: 9834372]
- Paz Y, Frolkis I, Pevni D, Shapira I, Yuhas Y, Iaina A, Wollman Y, Chernichovski T, Neshet N, Locker C, Mohr R, Uretzky G. Effect of tumor necrosis factor-alpha on endothelial and inducible nitric oxide synthase messenger ribonucleic acid expression and nitric oxide synthesis in ischemic and nonischemic isolated rat heart. *J Am Coll Cardiol* 2003; **42**: 1299-1305 [PMID: 14522499]
- Moreira AL, Sampaio EP, Zmuidzinis A, Frindt P, Smith KA, Kaplan G. Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. *J Exp Med* 1993; **177**: 1675-1680 [PMID: 8496685]
- Lopez-Talavera JC, Cadelina G, Olchowski J, Merrill W, Groszmann RJ. Thalidomide inhibits tumor necrosis factor alpha, decreases nitric oxide synthesis, and ameliorates the hyperdynamic circulatory syndrome in portal-hypertensive rats. *Hepatology* 1996; **23**: 1616-1621 [PMID: 8675185 DOI: 10.1002/hep.510230644]
- Souppison T, Yang S, Bernard C, Moreau R, Kirstetter P, D'Almeida M, Cailmail S, Tedgui A, Lebre D. Acute haemodynamic responses and inhibition of tumour necrosis factor-alpha by pentoxifylline in rats with cirrhosis. *Clin Sci (Lond)* 1996; **91**: 29-33 [PMID: 8774257]
- Austin AS, Mahida YR, Clarke D, Ryder SD, Freeman JG. A pilot study to investigate the use of oxpentifylline (pentoxifylline) and thalidomide in portal hypertension secondary to alcoholic cirrhosis. *Aliment Pharmacol Ther* 2004; **19**: 79-88 [PMID: 14687169]
- Laubach VE, Shesely EG, Smithies O, Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci USA* 1995; **92**: 10688-10692 [PMID: 7479866]
- Shesely EG, Maeda N, Kim HS, Desai KM, Kregg JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1996; **93**: 13365-13369 [PMID: 8844441 DOI: 10.1073/pnas.93.26.13365]

- Sci USA* 1996; **93**: 13176-13181 [PMID: 8917564]
- 25 **Golikov PP**, Nikolaeva Nlu. [Analysis of nitrites/nitrates (NOx) in serum]. *Biomed Khim* 2004; **50**: 79-85 [PMID: 15108630]
 - 26 **Alkam T**, Nitta A, Mizoguchi H, Saito K, Seshima M, Itoh A, Yamada K, Nabeshima T. Restraining tumor necrosis factor- α by thalidomide prevents the amyloid beta-induced impairment of recognition memory in mice. *Behav Brain Res* 2008; **189**: 100-106 [PMID: 18325608 DOI: 10.1016/j.bbr.2007.12.014]
 - 27 **Malleo G**, Mazzon E, Genovese T, Di Paola R, Muià C, Crisafulli C, Siriwardena AK, Cuzzocrea S. Effects of thalidomide in a mouse model of cerulein-induced acute pancreatitis. *Shock* 2008; **29**: 89-97 [PMID: 18246603]
 - 28 **Mazzon E**, Muià C, Di Paola R, Genovese T, De Sarro A, Cuzzocrea S. Thalidomide treatment reduces colon injury induced by experimental colitis. *Shock* 2005; **23**: 556-564 [PMID: 15897810]
 - 29 **Bellet P**, García-Pagán JC, Abalde JG, Bosch J. Primary prophylaxis of esophageal variceal bleeding in cirrhosis. *Gastroenterol Clin Biol* 2008; **32**: 532-540 [PMID: 18456445 DOI: 10.1016/j.gcb.2008.03.012]
 - 30 **Wang JJ**, Gao GW, Gao RZ, Liu CA, Ding X, Yao ZX. Effects of tumor necrosis factor, endothelin and nitric oxide on hyperdynamic circulation of rats with acute and chronic portal hypertension. *World J Gastroenterol* 2004; **10**: 689-693 [PMID: 14991939]
 - 31 **Cahill PA**, Redmond EM, Sitzmann JV. Endothelial dysfunction in cirrhosis and portal hypertension. *Pharmacol Ther* 2001; **89**: 273-293 [PMID: 11516480]
 - 32 **Enomoto N**, Takei Y, Hirose M, Kitamura T, Ikejima K, Sato N. Protective effect of thalidomide on endotoxin-induced liver injury. *Alcohol Clin Exp Res* 2003; **27**: 2S-6S [PMID: 12960498 DOI: 10.1097/01.ALC.0000078606.59842.01]
 - 33 **Park E**, Levis WR, Greig N, Jung E, Schuller-Levis G. Effect of thalidomide on nitric oxide production in lipopolysaccharide-activated RAW 264.7 cells. *J Drugs Dermatol* 2010; **9**: 330-333 [PMID: 20514789]
 - 34 **Hijikawa T**, Kaibori M, Uchida Y, Yamada M, Matsui K, Ozaki T, Kamiyama Y, Nishizawa M, Okumura T. Insulin-like growth factor 1 prevents liver injury through the inhibition of TNF- α and iNOS induction in D-galactosamine and LPS-treated rats. *Shock* 2008; **29**: 740-747 [PMID: 18004231 DOI: 10.1097/shk.0b013e31815d0780]
 - 35 **Marchi P**, Paiotti AP, Artigiani Neto R, Oshima CT, Ribeiro DA. Concentrated grape juice (G8000™) reduces immunorepression of iNOS, TNF- α , COX-2 and DNA damage on 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Environ Toxicol Pharmacol* 2014; **37**: 819-827 [PMID: 24637249 DOI: 10.1016/j.etap.2014.02.006]
 - 36 **Aires RD**, Capettini LS, Silva JF, Rodrigues-Machado Mda G, Pinho V, Teixeira MM, Cortes SF, Lemos VS. Paraquat poisoning induces TNF- α -dependent iNOS/NO mediated hyporesponsiveness of the aorta to vasoconstrictors in rats. *PLoS One* 2013; **8**: e73562 [PMID: 24039983 DOI: 10.1371/journal.pone.0073562]
 - 37 **Zheng Z**, Li Z, Chen S, Pan J, Ma X. Tetramethylpyrazine attenuates TNF- α -induced iNOS expression in human endothelial cells: Involvement of Syk-mediated activation of PI3K-IKK-I κ B signaling pathways. *Exp Cell Res* 2013; **319**: 2145-2151 [PMID: 23726836 DOI: 10.1016/j.yexcr.2013.05.018]
 - 38 **Medeiros R**, Prediger RD, Passos GF, Pandolfo P, Duarte FS, Franco JL, Dafre AL, Di Giunta G, Figueiredo CP, Takahashi RN, Campos MM, Calixto JB. Connecting TNF- α signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: relevance for the behavioral and synaptic deficits induced by amyloid beta protein. *J Neurosci* 2007; **27**: 5394-5404 [PMID: 17507561 DOI: 10.1523/JNEUROSCI.5047-06.2007]
 - 39 **Lee SK**, Huang H, Lee SW, Kim KH, Kim KK, Kim HM, Lee ZH, Kim HH. Involvement of iNOS-dependent NO production in the stimulation of osteoclast survival by TNF- α . *Exp Cell Res* 2004; **298**: 359-368 [PMID: 15265685 DOI: 10.1016/j.yexcr.2004.04.039]
 - 40 **Saud B**, Nandi J, Ong G, Finocchiaro S, Levine RA. Inhibition of TNF- α improves indomethacin-induced enteropathy in rats by modulating iNOS expression. *Dig Dis Sci* 2005; **50**: 1677-1683 [PMID: 16133968 DOI: 10.1007/s10620-005-2914-0]
 - 41 **Reichen J**. Portal hypertension: cytokines and endothelins. *Hepatogastroenterology* 1999; **46** Suppl 2: 1434-1436 [PMID: 10431705]
 - 42 **Lv P**, Luo HS, Zhou XP, Xiao YJ, Paul SC, Si XM, Zhou YH. Reversal effect of thalidomide on established hepatic cirrhosis in rats via inhibition of nuclear factor-kappaB/inhibitor of nuclear factor-kappaB pathway. *Arch Med Res* 2007; **38**: 15-27 [PMID: 17174718 DOI: 10.1016/j.arcmed.2006.09.006]
 - 43 **Hyakkoku K**, Nakajima Y, Izuta H, Shimazawa M, Yamamoto T, Shibata N, Hara H. Thalidomide protects against ischemic neuronal damage induced by focal cerebral ischemia in mice. *Neuroscience* 2009; **159**: 760-769 [PMID: 19166916 DOI: 10.1016/j.neuroscienc.2008.12.043]
 - 44 **Aukrust P**, Yndestad A, Damås JK, Ueland T, Øie E, Gullestad L. Potential mechanisms of benefit with thalidomide in chronic heart failure. *Am J Cardiovasc Drugs* 2007; **7**: 127-134 [PMID: 17503883]
 - 45 **Gullestad L**, Semb AG, Holt E, Skårdal R, Ueland T, Yndestad A, Frøland SS, Aukrust P. Effect of thalidomide in patients with chronic heart failure. *Am Heart J* 2002; **144**: 847-850 [PMID: 12422154]
 - 46 **Lafaras C**, Mandala E, Verrou E, Platogiannis D, Barbetakis N, Bischiniotis T, Zervas K. Non-thromboembolic pulmonary hypertension in multiple myeloma, after thalidomide treatment: a pilot study. *Ann Oncol* 2008; **19**: 1765-1769 [PMID: 18480066 DOI: 10.1093/annonc/mdn287]
 - 47 **Zhang SZ**, Li MT, He JG, Zeng XF. [Effects of three immunosuppressive agents and simvastatin on cell-proliferation and endothelin secretion of human endothelial cells]. *Zhonghua Neike Zazhi* 2008; **47**: 495-498 [PMID: 19040069]
 - 48 **Mitaka C**, Hirata Y, Ichikawa K, Yokoyama K, Emori T, Kanno K, Amaha K. Effects of TNF- α on hemodynamic changes and circulating endothelium-derived vasoactive factors in dogs. *Am J Physiol* 1994; **267**: H1530-H1536 [PMID: 7943398]
 - 49 **Osiovič H**, Goldberg RN, Suguihara C, Adams JA, Martinez O, Kuo G, Feuer WI, Offenbacher S, Bancalari E. Cardiopulmonary effects of tumor necrosis factor- α in the piglet: influence of cyclooxygenase inhibition. *Biol Neonate* 1995; **68**: 342-353 [PMID: 8835089]
 - 50 **Theodorakis NG**, Wang YN, Wu J, Maluccio MA, Skill NJ. Tumor necrosis factor alpha signaling in the development of experimental murine pre-hepatic portal hypertension. *Int J Physiol Pathophysiol Pharmacol* 2010; **2**: 104-110 [PMID: 21383890]
 - 51 **Cavo M**, Zamagni E, Cellini C, Tosi P, Cangini D, Cini M, Valdrè L, Palareti G, Masini L, Tura S, Baccarani M. Deep-vein thrombosis in patients with multiple myeloma receiving first-line thalidomide-dexamethasone therapy. *Blood* 2002; **100**: 2272-2273 [PMID: 12229885]
 - 52 **Niwayama S**, Loh C, Turk BE, Liu JO, Miyachi H, Hashimoto Y. Enhanced potency of perfluorinated thalidomide derivatives for inhibition of LPS-induced tumor necrosis factor- α production is associated with a change of mechanism of action. *Bioorg Med Chem Lett* 1998; **8**: 1071-1076 [PMID: 9871710]
 - 53 **Chang CC**, Wang SS, Huang HC, Lee FY, Lin HC, Lee JY, Chen YC, Lee SD. Chronic thalidomide administration enhances vascular responsiveness to vasopressin in portal-systemic collaterals of bile duct-ligated rats. *J Chin Med Assoc* 2009; **72**: 234-242 [PMID: 19467946 DOI: 10.1016/S1726-4901(09)70063-2]
 - 54 **Yang YY**, Huang YT, Lin HF, Lee FY, Lee KC, Chau GY, Loong CC, Lai CR, Lee SD. Thalidomide decreases intrahepatic resistance in cirrhotic rats. *Biochem Biophys Res Commun* 2009; **380**: 666-672 [PMID: 19285019 DOI: 10.1016/j.bbr.2009.01.160]
 - 55 **Moreau R**. VEGF-induced angiogenesis drives collateral circulation in portal hypertension. *J Hepatol* 2005; **43**: 6-8 [PMID: 15893843 DOI: 10.1016/j.jhep.2005.04.002]
 - 56 **Huang HC**, Wang SS, Hsin IF, Chang CC, Lee FY, Lin HC, Chuang CL, Lee JY, Hsieh HG, Lee SD. Cannabinoid receptor 2

agonist ameliorates mesenteric angiogenesis and portosystemic collaterals in cirrhotic rats. *Hepatology* 2012; **56**: 248-258 [PMID: 22290687 DOI: 10.1002/hep.25625]

57 **Yang YY**, Lee KC, Huang YT, Lee FY, Chau GY, Loong CC, Lin

HC, Lee SD. Inhibition of hepatic tumour necrosis factor- α attenuates the anandamide-induced vasoconstrictive response in cirrhotic rat livers. *Liver Int* 2009; **29**: 678-685 [PMID: 19413671 DOI: 10.1111/j.1478-3231.2009.01983.x]

P- Reviewer: Farias AQ, Sharma V **S- Editor:** Gou SX
L- Editor: Roemmele A **E- Editor:** Liu XM





Published by **Baishideng Publishing Group Inc**

8226 Regency Drive, Pleasanton, CA 94588, USA

Telephone: +1-925-223-8242

Fax: +1-925-223-8243

E-mail: bpgoffice@wjgnet.com

Help Desk: <http://www.wjgnet.com/esps/helpdesk.aspx>

<http://www.wjgnet.com>



ISSN 1007-9327

